

FORM PTO 1500 (REV 1-95)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY DOCKET NUMBER 2001_1463A	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371				U.S. APPLICATION NO. (If known, enter PCT No.) NEW 09/937296	
International Application No. PCT/GB00/01740		International Filing Date May 3, 2000		Priority Date Claimed May 10, 1999	
Title of Invention ASSAYS FOR NUCLEOSIDE DIPHOSPHATES AND TRIPHOSPHATES					
Applicant(s) For DO/EO/US Martin Hermann Klemens BRUNE, John Edgar Thomas CORRIE and Martin Ronald WEBB					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. §371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. §371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. §371(c)(2)) a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. ATTACHMENT A (WO 00/68418) c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US) 6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. §371(c)(2)). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)). a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19. 9. <input checked="" type="checkbox"/> An unexecuted oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). ATTACHMENT B 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).					
Items 11. to 14. below concern other document(s) or information included:					
11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. ATTACHMENT C 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. ATTACHMENT D <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 14. <input type="checkbox"/> Other items or information:					

THE COMMISSIONER IS AUTHORIZED
 TO CHARGE ANY DEFICIENCY IN THE
 FEE FOR THIS PAPER TO DEPOSIT
 ACCOUNT NO. 23-0575.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :
 Martin Hermann Klemens BRUNE et al. : **Attn: BOX PCT**
 Serial No. NEW : **Docket No. 2001_1463A**
 Filed September 25, 2001 :

ASSAYS FOR NUCLEOSIDE DIPHOSPHATES
 AND TRIPHOSPHATES
 [Corresponding to PCT/GB00/01740
 Filed May 5, 2000]

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,
 Washington, DC 20231

Sir:

Prior to calculating the filing fee, please amend the above-identified application as follows:

IN THE SPECIFICATION

Page 1, after the title, please insert:

This application is a 371 of PCT/GB00/01740 filed May 5, 2000.

IN THE CLAIMS

Please cancel without prejudice claims 1-19 and substitute therefor the following new claims:

20. A process for detecting the presence of a nucleoside diphosphate in a sample, comprising a step of detecting the dephosphorylation of the phosphoenzyme form a nucleoside diphosphate kinase (NDPK) by detecting a change in a characteristic of the NDPK which differs between its phosphorylated and unphosphorylated forms.

0937296-11401

21. A process for detecting the presence of a nucleoside triphosphate in a sample, comprising a step of detecting the phosphorylation of a nucleoside diphosphate kinase (NDPK) to the phosphoenzyme form by detecting a change in a characteristic of the NDPK which differs between its phosphorylated and unphosphorylated forms.

22. The process of claim 20 or claim 21, wherein the phosphorylation or dephosphorylation is detected by using an intrinsic property of NDPK.

23. The process of claim 20 or claim 21, wherein the NDPK is modified to carry a label which gives a different detectable signal when the enzyme is phosphorylated from when it is unphosphorylated.

24. The process of claim 23, wherein the NDPK carries a fluorescent label.

25. The process of claim 24, wherein the fluorescent label is attached to the NDPK via a cysteine residue.

26. The process of claim 24, wherein the fluorescent label is IDCC (*N*-[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboxamide).

27. The process of claim 20, wherein the nucleoside diphosphate is ADP or GDP.

28. The process of claim 21, wherein the nucleoside triphosphate is ATP or GTP.

29. The process of claim 20 or claim 21, being a quantitative process.

30. The process of claim 20 or claim 21, wherein the NDPK is the NDPK of *Myxococcus xanthus* carrying a Asp112-Cys mutation, and carrying an IDCC label at this mutated residue.

31. NDPK is modified to carry a label which gives a different detectable signal when the enzyme is phosphorylated from when it is unphosphorylated.

32. The NDPK of claim 31, wherein the label on the modified NDPK is a fluorescent label.

33. The NDPK of claim 32, wherein the fluorescent label is attached to the NDPK via a cysteine residue.

34. The NDPK of claim 32 or claim 33, wherein the fluorescent label is IDCC.

35. NDPK of *Myxococcus xanthus* carrying a Asp112-Cys mutation, and carrying an IDCC label at this mutated residue.

36. NDPK modified by the attachment of at least one detectable label that is sensitive to the binding of a nucleoside diphosphate.

37. A substrate having the NDPK of any one of claims 31, 35 or 36 immobilised thereto.

38. The NDPK of any one of claims 31, 35 or 36 for use as an *in vivo* or *in vitro* diagnostic reagent.

REMARKS


In the amendment, the Applicant has amended the specification to reflect the fact that this application is a national phase of the international application. In addition, the Applicant has cancelled without prejudice original claims 1-19 and added new claims 20-38, respectively. The wording of the new claims corresponds to the amendments to the claims effected during prosecution of the international application. In addition, the Applicant has revised the multiple dependencies of the claims to eliminate the improper multiple dependencies and to reduce the PTO filing fee.

Favorable action on the merits is solicited.

Respectfully submitted,

Martin Hermann Klemens BRUNE et al.

By


Warren M. Cheek, Jr.
Registration No. 33,367
Attorney for Applicants

WMC/dlk
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
September 25, 2001

ASSAYS FOR NUCLEOSIDE DIPHOSPHATES AND TRIPHOSPHATES

The contents of all documents cited herein are incorporated by reference in their entirety.

FIELD OF THE INVENTION

The invention relates to assays for nucleoside diphosphates, particularly ADP and GDP, and
5 assays for nucleoside triphosphates, particularly ATP and GTP.

BACKGROUND ART

Nucleoside diphosphates and triphosphates play important roles in biology. ADP is the immediate precursor for the formation of ATP, the universal currency of cellular energy. GDP is a substrate for succinyl CoA synthetase, a key enzyme of the Krebs cycle, and is formed during gluconeogenesis by phosphoenolpyruvate carboxykinase. It is also essential in G-protein signalling, microtubule growth, and visual excitation. UDP is involved in the epimerisation of galactose to glucose, the formation of sucrose, and in the growth of glycogen. CDP is an important group in the synthesis of phosphoglycerides. Nucleoside diphosphates are also the products of reactions catalysed by several major classes of enzymes, such as triphosphatases and kinases, and are therefore produced by many cellular processes, including motility, muscle contraction, DNA synthesis, transcription, translation and nitrogen fixation.

The detection and measurement of nucleoside diphosphates and triphosphates is thus important in the study of biology and metabolism, particularly in bioenergetics.

Assays for ADP and ATP in biological samples based on luciferase have been known for over
20 20 years [e.g. refs 1, 2, 3, 4]. Bioluminescent assays for ADP and ATP have been described for use in muscle and adipose tissue biopsies [5] and a three-enzyme bioluminescent system utilising luciferase has been reported for use in bacterial cell extracts [6]. A bioluminescent ADP assay optimised for use at high ATP:ADP ratios has been reported [7], but this requires the enzymatic removal of ATP. In general, it is easier to measure ATP in the presence of ADP
25 than to measure ADP in the presence of ATP.

Enzymatic spectrophotometric assays have also been described [e.g. 8].

Assays for GDP and GTP in biological samples are also well known [e.g. refs 9 & 10].

Reference 11 discloses column-based chromatographic assays for ADP, GDP, CDP and UDP. Radioactive assays for GDP and GTP have also been described [12, 13]. NMR-based assays for
30 measuring *in vivo* ADP levels are known for yeast [14], and NMR has also been used to measure ADP and ATP and erythrocytes [15].

DISCLOSURE OF THE INVENTION

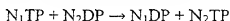
According to the present invention, nucleoside diphosphates are detected or measured by following the dephosphorylation of the phosphoenzyme form of nucleoside diphosphate kinase (NDPK). and nucleoside triphosphates are detected or measured by following the phosphorylation of NDPK to its phosphoenzyme form.

The invention thus provides (a) a process for detecting the presence of a nucleoside diphosphate in a sample, comprising the step of detecting the dephosphorylation of the phosphoenzyme form of a nucleoside diphosphate kinase, and (b) a process for detecting the presence of a nucleoside triphosphate in a sample, comprising the step of detecting the phosphorylation a nucleoside diphosphate kinase to the phosphoenzyme form.

The process will typically comprise the steps of:

- causing nucleoside diphosphate in sample to bind to NDPK phosphoenzyme. or causing nucleoside triphosphate in sample to phosphorylate NDPK; and
- detecting a change in a characteristic of the enzyme which differs between its phosphorylated and unphosphorylated forms.

The term "NDPK" means an enzyme having the activity of the enzyme classified as EC 2.7.4.6, namely the transfer of the γ -phosphate group of a nucleoside triphosphate (N_1TP) to a nucleoside diphosphate (N_2DP) via a ping-pong mechanism:



Based on this reaction scheme, the systematic name of NDPK is "ATP:nucleoside-diphosphate phosphotransferase". but the common name is "nucleoside diphosphate kinase". The enzyme has also been variously described as: kinase (phosphorylating), nucleoside diphosphate; nucleoside 5'-diphosphate kinase: nucleoside diphosphate (UDP) kinase: nucleoside diphosphokinase: nucleotide phosphate kinase: NM23.

NDPKs have been described for a number of organisms, both prokaryotic and eukaryotic *e.g.* human, cows, monkeys, mice, *Xenopus*, oats, peas, potatoes, yeast, *Bacillus subtilis*, *E.coli*, *Myxococcus xanthus*, avian myeloblastosis virus *etc.* These differ by cellular location, molecular weight, oligomeric structure, isoelectric point, reaction kinetics, substrate preference, pH optimum, pH range, temperature optimum, cation requirements (Mn^{2+} , Mg^{2+} , Co^{2+} , Ca^{2+} *etc.*), and various isoforms have been described. Given the variety of suitable enzymes available, the skilled person can easily select and purify a NDPK to suit any particular situation.

The NDPK enzyme uses a ping-pong mechanism, transferring the γ -phosphate from a nucleoside triphosphate (N_1TP) to an active site histidine to form a phosphoenzyme intermediate, and then to a nucleoside diphosphate (N_2DP). The invention is based on the finding that the phosphoenzyme intermediate is stable over a time-scale that allows its detection and measurement. Other enzymes that phosphorylate nucleoside diphosphates via a phosphoenzyme intermediate, preferably with a single binding site for nucleotide, may also be used in the invention.

The phosphoenzyme is able to transfer its phosphate group to N_2DP in a sample to form the corresponding N_2TP . Detection of this transfer can therefore be used for the detection of nucleoside diphosphate. To detect nucleoside diphosphate according to the invention, therefore, phosphoenzyme is required as a reagent. This can be readily formed by, for example, incubating NDPK with excess NTP, typically ATP. Formation of phosphoenzyme in this way is facilitated by removing Mg^{2+} [16], for instance by using EDTA. Chemical phosphorylation of histidine using phosphoramidate as a phosphorylating agent may also be used [17].

The phosphoenzyme can be isolated for use as a reagent. It has been found that the phosphoenzyme can be stored on ice for over 48 hours without dephosphorylation, and can be stored for longer periods (at least 5 months) at $-80^\circ C$ (although repeated freeze-thawing results in some dephosphorylation). The stability of the phosphoenzyme over the time range needed for its preparation, and subsequently for monitoring kinetic events such as the release of ADP from an ATPase, is particularly advantageous.

When added to a sample containing NDP, the phosphoenzyme is dephosphorylated by the transfer of its phosphate group to the NDP. When added to a sample containing NTP, the phosphoenzyme is formed by the transfer of the NTP γ -phosphate group to the enzyme. The invention relies on the ability to distinguish between the phosphorylated and dephosphorylated forms of NDPK.

In order to distinguish the phosphorylated and unphosphorylated forms of NDPK, any suitable measurable change can be used.

For instance, intrinsic properties of the enzyme can be used. Depending on the particular NDPK chosen, the following methods are examples of how dephosphorylation/phosphorylation may be detected, with varying levels of sensitivity:

- The location of a phosphate (*i.e.* either bound to NDPK, or as the γ -phosphate of a NTP) can be ascertained by following the ^{31}P NMR spectrum.
 - Protons whose environment changes upon dephosphorylation can be detected by, for instance, NMR.
 - 5 - Dephosphorylation may cause a change in the fluorescence of a tryptophan residue in the protein [*e.g.* ref 18].
 - Dephosphorylation can be detected by following the loss of ^{32}P from radio-labelled phosphoenzyme. The radio-isotope can be conveniently incorporated into NDPK by using [γ - ^{32}P]ATP.
 - 10 - Circular dichroism, or any other suitable spectrometric technique, can detect conformational changes which occur on dephosphorylation.
 - Dephosphorylation may result in a change in surface plasmon resonance properties.
- Rather than using properties inherent in the wild-type enzyme, it may be desired to modify the enzyme in some way. This may also be important where dephosphorylation of the NDPK of choice does not exhibit an intrinsic measurable change which can be readily followed.
- 15 One particularly preferred modification is the addition of a fluorescent label to the enzyme, typically via a cysteine residue. If the wild-type protein lacks a suitable cysteine residue (*e.g.* the NDPK of *Myxococcus xanthus*), this can easily be introduced by mutagenesis [*e.g.* 19]. A suitable position for mutation can easily be determined by the skilled person, whilst ensuring
- 20 that the mutation does not disrupt the enzymatic activity [*e.g.* 20]. At any given amino acid residue, particular labels may give better results than others. Suitable combinations of label and residue can be determined by routine experimentation.

Preferred fluorescent labels are based around coumarin. Particularly preferred is *N*-[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboxamide [21; Fig. 1], referred to simply as 'IDCC' hereafter. This is preferably attached to a cysteine residue, and preferably exhibits a high fluorescence when NDPK is phosphorylated, and a low fluorescence when NDPK is dephosphorylated. When suitably attached to NDPK, this label offers the advantage that the phosphoenzyme can detect small quantities of ADP in the presence of much higher concentrations of ATP. This is extremely important for experiments in situations where ATP

30 levels are high *e.g.* in single muscle fibres. It is also able to respond very quickly to changes in ADP levels, and gives a large signal change over a range of several hundred micromolar.

Other labels which can be introduced in similar ways include ESR labels, luminescent labels, phosphorescent labels, and other suitable chromophores.

It will be appreciated that some of the various options available to the skilled person are more suitable than others for detecting the phosphorylation/dephosphorylation of the enzyme in real-time. Fluorescence is highly suitable for real-time detection, whereas methods such as those using radio-labels are more suitable for measuring end-points.

The nucleoside diphosphate/triphosphate which is assayed must be a substrate of the NDPK being utilised. Various NDP substrates have been described [e.g. 22, 23, 24] including ADP, CDP, GDP, UDP, IDP, XDP, their deoxy-derivatives (e.g. dADP, dCDP, dGDP, dTDP, dUDP), 6-aza-UDP, 8-bromo-IDP, 8-aza-GDP, and 8-aza-UDP, and adenosine 5'-methylene diphosphonate. Each of these compounds is phosphorylated by the phosphoenzyme (with varying reaction affinities and kinetics, depending on both the NDPK and the substrate being utilised), and can thus be assayed according to the invention.

The invention is preferably used to detect and measure ADP or GDP. Accordingly, a NDPK may be chosen which shows a preference towards one of these substrates.

In preferred embodiments of the invention, the detection process gives quantitative data, that is to say the invention provides a process for quantifying nucleoside diphosphate or triphosphate in a sample. This will typically involve the step of relating a change in the detectable characteristics of a NDPK to a concentration of NDP or NTP. It will be appreciated that this may require a calibration to be performed (e.g. for measuring dephosphorylation via a fluorescent label such as IDDC) or comparison with a standard. Calibration will typically be performed for the desired range of concentrations to be measured.

In a first quantitative aspect, the amount of nucleoside diphosphate or nucleoside triphosphate is determined by measuring the decrease (NDP) or increase (NTP) in the level of phosphoenzyme after the addition of phosphoenzyme (NDP) or unphosphorylated enzyme (NTP) to a sample.

In a second quantitative aspect, the rate of production of nucleoside diphosphate or triphosphate can be determined by following the decrease (NDP) or increase (NTP) in the level of phosphoenzyme over time. By fitting the measured values to a suitable mathematical model (e.g. a simple model based on first-order exponential decrease), the rate of nucleoside diphosphate or triphosphate production can be determined. In this aspect, dephosphorylation of

the phosphoenzyme (NDP) or phosphorylation of the enzyme (NTP) is preferably measured using a real-time detection method.

The process of the invention is preferably suitable for use either *in vivo* or *in vitro*. The method is preferably suitable for *in situ* use in a muscle fibre, and is preferably gives data suitable for calculating the rate of ADP release from actomyosin.

As well as the step of detecting the dephosphorylation of the phosphoenzyme (NDP-related aspects), the process will usually comprise the initial step of adding NDPK phosphoenzyme to a sample of interest. This may be preceded by the preparation of phosphoenzyme from unphosphorylated NDPK.

The process may also include a step of analysing any data obtained during the process, such as fitting the data to an equation in order to derive quantitative values.

The process preferably avoids the use of reagents such as theophylline, desdanine and Ag^+ , which may inhibit the NDPK activity.

As well as the above processes, the invention provides reagents for use in the processes.

The invention provides NDPK which is modified to carry a label which gives a different detectable signal when the enzyme is phosphorylated from when it is unphosphorylated.

The label on the modified NDPK may be a fluorescent group, preferably IDCC.

The label will typically be attached to an amino acid residue in the enzyme. It is preferred to attach the label to a cysteine residue.

A particularly preferred reagent is the NDPK of *M.xanthus* carrying a Asp112→Cys mutation, and carrying an IDCC label at this mutated residue. This reagent as a phosphoenzyme is about three orders of magnitude more sensitive to ADP than to ATP.

The invention also provides a NDPK modified by the attachment of at least one detectable label that is sensitive to the binding of a nucleoside diphosphate.

The invention also provides substrates having these NDPK reagents immobilised thereto. These include columns or beads. This may be used in combination with ^{32}P -phosphoenzyme, such that ADP in a sample incubated with immobilised NDPK will become radio-labelled in its conversion to ATP. Radioactivity in free solution will therefore indicate the amount of ADP in the original sample

The invention also provides processes for the production of these NDPK reagents.

Furthermore, the invention provides these NDPK reagents for use as *in vivo* or *in vitro* diagnostic reagent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of a preferred fluorescent label, IDCC.

Figure 2 shows the emission spectra of IDCC-NDPK in the presence of (A) ADP/ATP and (B) GDP/GTP.

Figure 3 shows a titration of IDCC-labelled phosphoenzyme with ADP/GDP (open symbols \circ and \square) and ATP/GTP (closed symbols \bullet and \blacksquare). Figure 4 shows the same experiments using unphosphorylated enzyme.

Figure 5 shows a calibration curve obtained in a stopped flow experiment, indicating how fluorescence change varies with ADP concentration.

Figure 6 shows transient kinetic data obtained using stopped-flow fluorescence. The dashed curves represent the best fit to a single exponential equation. 6A & 6B show data obtained with ADP and ATP, respectively. 6C shows a plot of observed rate constants against ADP (\circ) or ATP (\bullet) concentration. The solid line shows the best fit to a linear equation with the respective deduced second-order rate constants. The dashed line and squares (\blacksquare) show observed rate constants that were obtained when the ADP titration was repeated in the presence of 1mM ATP.

Figure 7 shows a plot obtained from a single rabbit psoas muscle fibre (skinned), indicating the increase in muscle tension and the accompanying decrease in fluorescence due to dephosphorylation of IDCC-NDPK~P.

Figure 8 shows a plot obtained using human rho protein, indicating the release of GDP into solution. Data were fitted to a single-exponential plus linear equation, shown as a solid line.

EXAMPLES

General molecular biology techniques were carried out according to Sambrook *et al.* [25].

Preparation of NDPK

The NDPK of *M.xanthus* is encoded by the *ndk* gene, which has been cloned and expressed in *E.coli* [26]. The protein is a homo-tetramer of 16kDa subunits, it has been characterised, and a

crystal structure has been determined [16,27]. The wild-type sequence does not contain any cysteine, so the gene was manipulated to introduce cysteine residues by site-directed mutagenesis in *E. coli* strains TG1 and DH5 α using either a phosphothioate-based method [28, produced in kit form by Amersham] or the PCR-based QuikChange kit [Stratagene].

- 5 Using the Amersham kit, the 0.8 kb *HindIII-EcoRI* fragment of pJM5C2A [29] containing the *ndk* gene from *M.xanthus* was ligated with M13mp19, and the resulting recombinant clones were used to provide single-stranded DNA templates for mutagenesis. For cloning, the mutated *ndk* genes were cloned back into pJM5C2A. As an alternative, the 0.7 kb *BstXI-EcoRI* fragment of the M13ndk constructs was ligated into a modified form of the InvitrogenTM pRSetA
10 expression vector, whose coding sequence for a histidine tag fused to the N-terminus of NDPK had been removed. This yielded the 3.5 kb pRSndkX series of plasmids, where the final "X" is a number in a series of *ndk* mutations.

pRSndk was also used as a template for the QuikChange method.

- Various mutant proteins containing cysteine residues were prepared, including D112C (*i.e.*
15 Asp-112 was mutated to Cys) and D62C. Positions for mutation were typically chosen on the basis of their proximity to the nucleotide-binding cleft seen in the crystal structure [16].

- The mutant D112C gene was produced in plasmid pRSndk4, which was also used for expression. For best results, freshly-transformed cells were used for starter cultures. 200 μ l calcium-competent BL21 cells [Novagen] were incubated with 2ng pRSndk4 plasmid DNA for
20 30 minutes on ice. Half of this mixture was then spread onto an LB agar plate containing 0.1mg/ml ampicillin and incubated overnight at 37°C. Plates typically contained 50-100 colonies. 100ml LB medium containing 0.1mg/ml ampicillin was inoculated with 2-3 colonies from the plate and grown for 9 hours at 37°C until cells had just entered stationary phase. For the main culture, 8x500ml LB+ampicillin was inoculated with 10ml started culture and
25 incubated at 37°C for 6 hours, after which time the cells had typically reached OD₅₉₅ of 0.38. At this point, 0.5mg/ml IPTG was added to each flask, and the cells grown for a further 16 hours. Cells were harvested by centrifugation in a Beckman L2 centrifuge at 3800rpm and 20°C for 20 minutes. The pellet was resuspended in 100ml Buffer A (20mM Tris-HCl, pH 8.2, 1mM EDTA) and stored at -80°C.

Approx. 35ml of the frozen cell suspension was lysed by slow thawing and then sonication. The supernatant (*i.e.* crude extract) was retained. Expression levels were determined by SDS-PAGE. NDPK^{D112C} made up >50% cytosolic protein.

NDPK purification involved two column chromatography steps. First, after adjusting pH and ionic strength using Buffer A + 10mM DTT, the crude extract (approx. 200ml) was loaded onto a 120ml Q-Sepharose ion-exchange column at 4°C and a flow rate of 2.0ml/min. The column was washed with 1 volume buffer and eluted with a continuous linear gradient (500ml) from 0-0.3M NaCl at 1ml/min flow rate. The bulk of NDPK (about 80%) does not bind to the resin under these conditions.

After concentration using an Amicon YM10 ultrafiltration membrane, the eluate was loaded onto a G-100 Sepharose gel filtration column in Buffer A. At a flow-rate of 1.0ml/min, the elution profile showed 2 peaks. The first contained DNA, and the second contained proteins. Fractions containing NDPK were pooled and concentrated as before.

It was confirmed that there was no contamination with *E.coli* NDPK, which has a similar sequence and is also a tetramer [30]. Mass spectrometry data showed that the purified protein was a single species with MW 15993±1 Da, matching the calculated MW of the mutant protein minus the N-terminal Met, which was shown to be missing when expressed in *E.coli* [16]. The yield of pure NDPK^{D112C} from a 4-litre culture was around 300mg.

Fluorescent labelling of NDPK

NDPK^{D112C} was incubated with a 2.5-fold excess of IDCC (Fig. 1) in 50mM Tris/HCl, pH 8.1 for 1 hour at 37°C. In a typical experiment, the volume of the labelling solution was 3ml and the concentration of NDPK^{D112C} was 150µM. After incubation, this solution was filtered through a 0.2µm Acrodisc filter (Gelman) and loaded onto a PD10 desalting column (Pharmacia) equilibrated in Buffer B (10mM Tris-HCl, pH 8.0, 1mM EDTA). The eluate (approx. 6ml) containing labelled protein was immediately loaded onto a 25ml Q-Sepharose column equilibrated in Buffer B, followed by a 20ml linear gradient from 0-0.1M NaCl. The labelled protein was completely eluted before the gradient was applied, and was concentrated in the same way as unlabelled protein. The concentration of the protein was determined using absorbance spectroscopy, assuming that the absorbance spectrum of the fluorophore was unaltered ($\epsilon_{430nm}^{46800} \text{ M}^{-1}\text{cm}^{-1}$). This concentration value agree with that measured by a colorimetric assay using a bovine serum albumin standard curve. After correcting for the absorbance of IDCC at 276nm (0.198 of that at 430nm in the dithiothreitol adduct), the molar extinction coefficient for NDPK could be calculated as $\epsilon_{276nm}^{7600} \text{ M}^{-1}\text{cm}^{-1}$.

The yield after labelling and purification was typically 65%. This high yield indicates that the thiol group of Cys-112 is easily accessible. Mass spectrometry revealed that one molecule IDCC was incorporated per protein molecule, with no indication of second-site labelling, and the mass was that expected for IDCC-NDPK.

- 5 To check that mutation and labelling did not affect oligomerisation, the MW of the complex was determined by sedimentation equilibrium centrifugation. The results indicate that IDCC-NDPK is a tetramer of MW 62.7kDa (4% lower than that calculated for a tetramer from mass spectrometry data).

Where rapid analysis of the fluorescent properties of a new mutant/fluorophore combination
10 was required, labelling was performed on a smaller scale, typically using a NDPK concentration of 100-200µl and avoiding the ultrafiltration and Q-Sepharose steps.

Other thiol-reactive environmentally-sensitive fluorescent labels which were tested included MDCC (*N*-[2-(1-maleimidyl)ethyl]-7-diethylaminocoumarin-3-carboxamide) [21].

Phosphorylation

- 15 IDCC-NDPK was incubated with ATP, typically at a 5-fold excess, but ≤ 1 mM. The incubation was for 30-45 minutes at 37°C in either in 10mM Tris/HCl, pH 8.0, 1mM EDTA, or in 10mM PIPES, pH 7.0, 1mM EDTA. The protein was separated using a PD10 column equilibrated in the same buffer. Typically, more than 90% of the eluted protein was phosphoenzyme. The solution was either used immediately or after storage on ice for up to 48 hours. If necessary, the
20 solution was concentrated using a microcentrifuge concentrator.

Fluorescent properties

The spectroscopic properties of the phosphoenzyme (IDCC-NDPK~P) were compared with the unphosphorylated form of the enzyme. Data was recorded on a Perkin-Elmer LS50B luminescence spectrometer with a xenon lamp and monochromator slit widths set to 2.5 or 5nm.

- 25 The fluorescence of IDCC-NDPK was 4-fold greater in the presence of 50µM ATP than in the presence of 50µM ADP (Figure 2A). Similar results were obtained with 100µM GDP/GTP (Figure 2B). In both cases the spectra were recorded at 20°C from a 200µl solution of 2µM protein in 20mM PIPES, pH 7.0, 2mM MgCl₂. Excitation was at 441nm, slit width 5nm. No correction was made for the small (1%) volume change. These results indicate that
30 IDCC-NDPK~P has a high fluorescence compared with the unphosphorylated form.

A titration of a solution of 2 μ M IDCC-NDPK-P with ADP showed a large decrease in the fluorescence signal (Figure 3A), whereas there was no change with ATP over the same concentration range (<100 μ M). Similar data were obtained with GDP/GTP (Figure 3B). The ADP/ATP assays were carried out at 21°C in 10mM Tris/HCl, pH 8.0, 5mM MgCl₂. Excitation was at 432nm (5nm slit width) and emission was recorded at 478nm (2.5nm slit width). The GDP/GTP assays were carried out at 20°C in 20mM PIPES, pH 7.0, 2mM MgCl₂. Excitation was at 441nm (5nm slit width) and emission was recorded at 475nm (5nm slit width). In both cases data were corrected for the small volume increases. The small decrease at the first addition of GTP in Figure 3B is due to partial hydrolysis of GTP in the diluted 200 μ M stock solution; subsequent GTP additions were from a 2mM stock solution.

Together with data from unphosphorylated enzyme (Figures 4A and 4B), this suggests that most of the fluorescence change is due to phosphorylation/dephosphorylation of the enzyme.

Increasing ADP above 50 μ M produces a small fluorescence increase. This is probably due to a non-specific interaction between ADP and NDPK, as suggested for non-labelled wild-type enzyme [18]. The change in fluorescence signal is linear with [ADP] up to at least 50% of the protein concentration.

To derive quantitative data from the fluorescence measurements, the calibration curve shown in fig. 5 was plotted. The calibration data were obtained in a stopped flow experiment by mixing 10 μ M IDCC-NDPK-P and 50 μ M ATP with 0, 2.5, 5.0, 7.5 or 10.0 μ M ADP (10mM PIPES buffer, pH 7.0, 1mM MgCl₂, 20°C). The amplitudes of the fluorescence change (ΔF) were averaged from two experiments at each concentration and plotted against ADP concentration.

There is a shift of 6nm in the absorbance maxima of the fluorophore between IDCC-NDPK and IDCC-NDPK-P, but only 2nm in the emission maxima. The fluorescence quantum yields for the two forms of the labelled enzyme were determined using as reference the known value of 0.83 for Coumarin 314 in ethanol [31]. There was a 4-fold decrease in the quantum yield when going from phosphorylated (0.22) to unphosphorylated enzyme (0.054), in good agreement with the observed change in emission intensity (Figure 2), although the quantum yield for the phosphoenzyme was still significantly lower than that for Coumarin 314 in buffer (0.52).

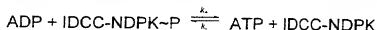
Transient kinetics

Three different transient kinetic measurements were carried out in a stopped-flow apparatus.

Firstly, the rate of IDCC-NDPK~P dephosphorylation was measured over a range of ADP concentrations. Figure 6A shows the emission data for mixing 50μM (a) and 250μM (b) ADP with 1μM protein in 20mM PIPES, pH 7.0, 2mM MgCl₂. The observed rate constants (a: 11.1±0.01 s⁻¹; b: 57.2±0.07s⁻¹) increased linearly in proportion to the increasing ADP concentrations, and a second order rate constant was calculated from the slope of the linear fit, giving 0.21x10⁶ M⁻¹s⁻¹ (Figure 6C).

In a second experiment, the rate of phosphoenzyme production was measured by mixing IDCC-NDPK~P enzyme with ATP (Figure 6B). Again, there was a linear increase in rates with increasing ATP (5.57±0.01 s⁻¹ at 50μM ATP; 18.4±0.03 s⁻¹ at 250μM ATP), and the deduced second order rate constant was 0.072x10⁶ M⁻¹s⁻¹ (Figure 6C).

In a third experiment, the rate of dephosphorylation was measured in the presence of varying concentrations of ATP. The observed rates were higher but the intensity changes lower in the presence of ATP than in its absence. From the slope of the dashed line in Figure 6C (■), the second order rate constant is calculated as 0.135x10⁶ M⁻¹s⁻¹ in the presence of ATP. The increase in rate produced by ATP can be explained by considering the enzymatic equilibrium:



The observed rate is $k_+[ADP] + k_-[ATP]$, and therefore increases with both $[ADP]$ and $[ATP]$.

This rate acceleration is advantageous in muscle fibres, for instance, where ATP levels are usually at least 1mM. Dephosphorylation of the phosphoenzyme should be very fast, even at ADP concentrations which are initially low.

Measurements in a single muscle fibre

An experiment was performed on a single rabbit psoas muscle fibre in the presence of Ca²⁺, essentially as described in reference 32, with the following modifications. An incubation of the fibre for 10 minutes in rigor solution containing 8U/ml Apyrase to remove background ADP was followed by 10 minutes incubation in loading solution containing 380μM IDCC-NDPK~P and 5mM NPE-caged ATP [the P²-1-(2-nitrophenyl)ethyl ester of ATP]. The fibre was transferred to silicone oil, and ATP (approx. 1 mM) was released by laser flash photolysis (347nm) at time zero. The temperature during the experiment was 18°C, and the aqueous solutions were pH 7.1 and pCa 4.5. Fluorescence excitation was at 425nm, and emission was detected through a long-pass filter (450-650nm).

The fluorescence trace (bottom curve, Figure 7) after time zero consists of three parts: first, a flash artefact; then, a lag, during the time for the first ATPase cycle to occur as far as bound ADP; finally, a slow phase of decreasing fluorescence, representing the reaction of

IDCC-NDPK~P with ADP. The initial slope of this curve gives the rate of ADP release from actomyosin as 11s^{-1} .

Similar experiments in muscle fibres using NPE-caged ADP to measure ADP production independently from myosin ATPase show that (i) the phosphoenzyme is able to maintain its sensitivity and reactivity for ADP when it is within a muscle fibre (ii) a signal is detectable for ADP released from myosin ATPase (iii) the muscle fibre is developing tension in the expected manner.

Nucleotide exchange from human Rho protein

The rate of nucleotide exchange from a small G protein, rho (which also has GTPase activity), was determined in solution. The rate of hydrolysis was also obtained indirectly.

Human rho protein was prepared in a form containing tightly-bound GDP. In the presence of excess added GTP, nucleotide exchange occurs such that GDP is released into free solution where it can be measured according to the invention. Once GTP is bound to rho, its hydrolysis is slowly catalysed by the protein at a steady-state rate. This creates more GDP, which is measured once it is released.

Fluorescence emission was recorded for a 200 μl solution containing 20mM Tris/HCl, pH 7.6, 1mM MgCl_2 , 100mM $(\text{NH}_4)_2\text{SO}_4$, 10 μM GTP, and 2 μM IDCC-NDPK~P. The reaction was started at $t=0$ by adding 2 μM Rho.GDP. Excitation was at 440nm with the emission monochromator set to 475nm. The temperature was 30°C. The data were normalised using the fluorescence change for 1 μM GDP obtained from a titration of IDCC-NDPK~P with different GTP/GDP ratios. The data (figure 8) were fitted to a single-exponential plus linear equation with a rate constant of 0.196min^{-1} for the exponential phase and a slope of $41.2\text{nM GDP min}^{-1}$ ($3.43 \times 10^{-4}\text{s}^{-1}$, after dividing by $[\text{Rho}]$) for the linear phase. The exponential "burst" represents the exchange of GDP bound to rho for GTP. The linear phase represents steady state: exchange of further GTP onto rho is limited by hydrolysis to GDP.

The rates were also measured by 'standard' means, and similar results were found.

It will be understood that the invention is described above by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

REFERENCES (the contents of which are incorporated herein in full)

1. Holmsen *et al.* (1972) *Anal. Biochem.* 46:489-501
2. Kimmich *et al.* (1975) *Anal Biochem* 69:187-206
3. Loxdale (1976) *J Physiol (Lond)* 240:4P-5P
4. Spielmann *et al.* (1981) *Anal Biochem* 113:172-178
5. Feradui *et al.* (1981) *Int J Sports Med* 2:106-109
6. Brovko *et al.* (1994) *Anal Biochem* 220:410-414
7. Schultz *et al.* (1993) *Anal Biochem* 215:302-304
8. Tornheim & Schultz (1993) *Anal Biochem* 211:329-330
9. Pogson *et al.* (1979) *Int J Biochem* 10:995-1000
10. de Azeredo *et al.* (1979) *Anal Biochem* 95:512-519
11. Sato *et al.* (1983) *Anal Biochem* 135:431-435
12. Goswami & Pande (1984) *J Biochem Biophys Methods* 9:143-151
13. Cerpovicz & Ochs (1991) *Anal Biochem* 192:197-202
14. Brindle *et al.* (1990) *Biochem* 29:3295-3302
15. Petersen *et al.* (1990) *Biochim Biophys Acta* 1035:169-174
16. Williams *et al.* (1993) *J Mol Biol* 234:1230-1247.
17. Morera *et al.* (1995) *Biochemistry* 34:11062-11070.
18. Schaertl *et al.* (1998) *J Biol Chem* 273:5662-5669
19. Sundin *et al.* (1996) *J. Bacteriol* 178:7120-7128
20. Izumiya & Yamamoto (1995) *J Biol Chem* 270:27859-27864.
21. Corrie (1994) *J Chem Soc. Perkin Trans* 1:2975-2982.
22. Parks & Agarwal in *The Enzymes*, 3rd ed (ed. Boyer). 8:307-333
23. Agarwal *et al.* (1978) *Methods Enzymol* 51:376-386
24. Jong & Ma (1991) *Arch Biochem Biophys* 291:241-246.
25. Sambrook *et al.* (1989) *Molecular cloning - a laboratory manual*.
26. Muñoz-Dorado *et al.* (1990) *J Biol Chem* 265:2702-2706.
27. Muñoz-Dorado *et al.* (1990) *J Biol Chem* 265:2707-2712.
28. Olsen *et al.* (1993) *Meth Enzymol* 217:189-217.
29. Kindly provided by Dr R Williams. Cambridge.
30. Almaula *et al.* (1995) *J Bacteriol* 177:2524-2529.
31. Fletcher & Bliss (1978) *Appl Phys* 16:289-295.
32. He *et al.* (1997) *J. Physiol.* 501.1:125-148.

CLAIMS (Article 34 PCT)

1. A process for detecting the presence of a nucleoside diphosphate in a sample, comprising the step of detecting the dephosphorylation of the phosphoenzyme form of a nucleoside diphosphate kinase (NDPK) by detecting a change in a characteristic of the NDPK which differs between its phosphorylated and unphosphorylated forms.
2. A process for detecting the presence of a nucleoside triphosphate in a sample, comprising the step of detecting the phosphorylation of a nucleoside diphosphate kinase (NDPK) to the phosphoenzyme form by detecting a change in a characteristic of the NDPK which differs between its phosphorylated and unphosphorylated forms.
3. The process of claim 1 or claim 2, wherein the phosphorylation or dephosphorylation is detected by using an intrinsic property of NDPK.
4. The process of claim 1 or claim 2, wherein the NDPK is modified to carry a label which gives a different detectable signal when the enzyme is phosphorylated from when it is unphosphorylated.
5. The process of claim 4, wherein the NDPK carries a fluorescent label.
6. The process of claim 5, wherein the fluorescent label is attached to the NDPK via a cysteine residue.
7. The process of claim 5 or claim 6, wherein the fluorescent label is IDCC (*N*-[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboxamide).
8. The process of claim 1, wherein the nucleoside diphosphate is ADP or GDP.
9. The process of claim 2, wherein the nucleoside triphosphate is ATP or GTP.
10. The process of any preceding claim, being a quantitative process.
11. The process of any preceding claim, wherein the NDPK is the NDPK of *Myxococcus xanthus* carrying a Asp112→Cys mutation, and carrying an IDCC label at this mutated residue.
12. NDPK which is modified to carry a label which gives a different detectable signal when the enzyme is phosphorylated from when it is unphosphorylated.
13. The NDPK of claim 12, wherein the label on the modified NDPK is a fluorescent label.

-2-

14. The NDPK of claim 13, wherein the fluorescent label is attached to the NDPK via a cysteine residue.

15. The NDPK of claim 13 or claim 14, wherein the fluorescent label is IDCC.

16. NDPK of *Myxococcus xanthus* carrying a Asp112→Cys mutation, and carrying an IDCC
5 label at this mutated residue.

17. NDPK modified by the attachment of at least one detectable label that is sensitive to the binding of a nucleoside diphosphate

18. A substrate having the NDPK of any one of claims 12 to 17 immobilised thereto.

19. The NDPK of any one of claims 12 to 17 for use as an *in vivo* or *in vitro* diagnostic
10 reagent.

1/7

Fig. 1

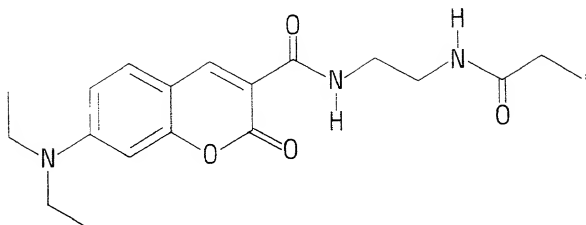
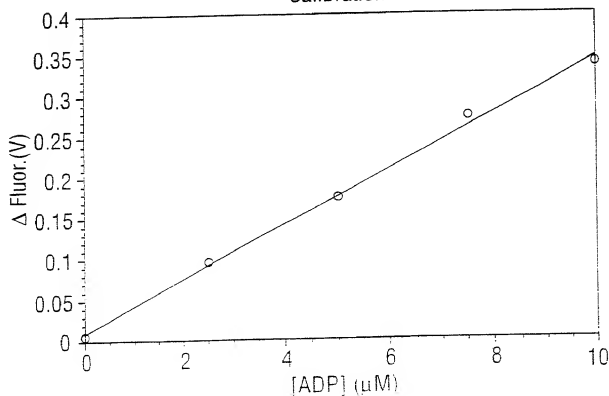


Fig. 5

Calibration



2/7

Fig. 2A

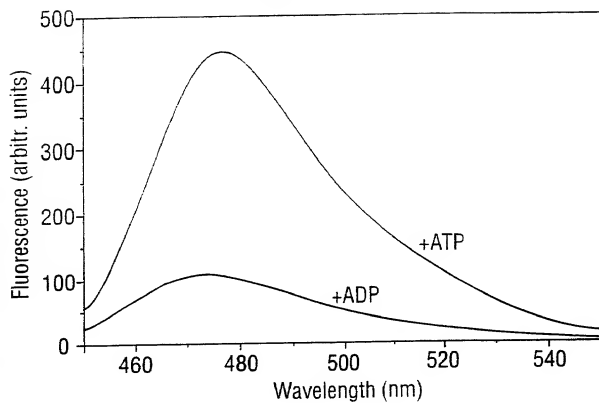
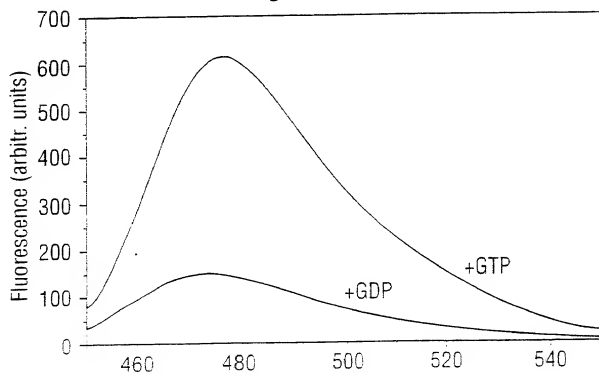


Fig. 2B



3/7

Fig. 3A

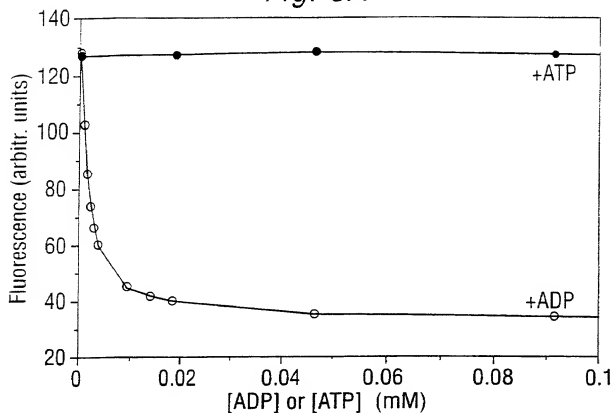
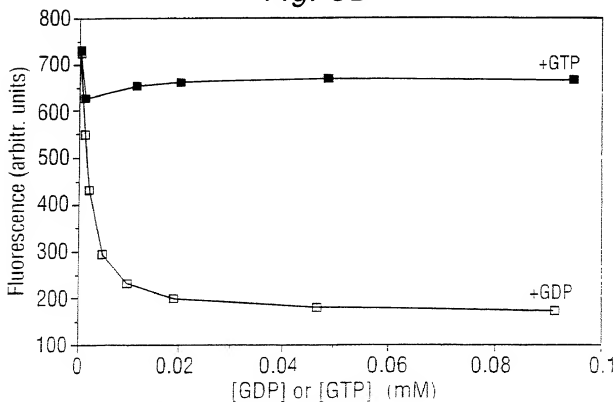


Fig. 3B



4/7

Fig. 4A

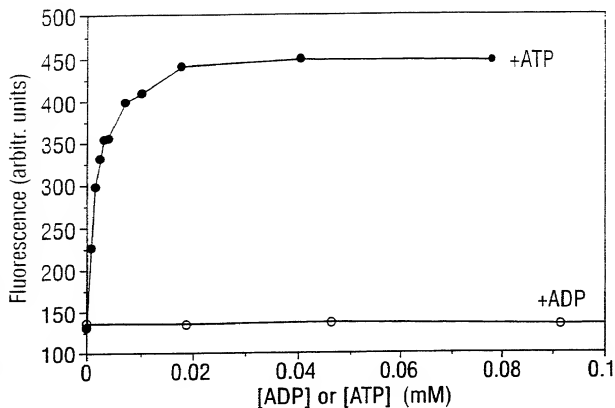
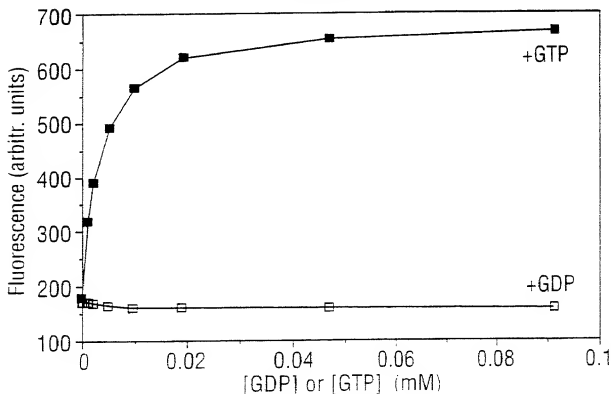


Fig. 4B



5/7

Fig. 6A

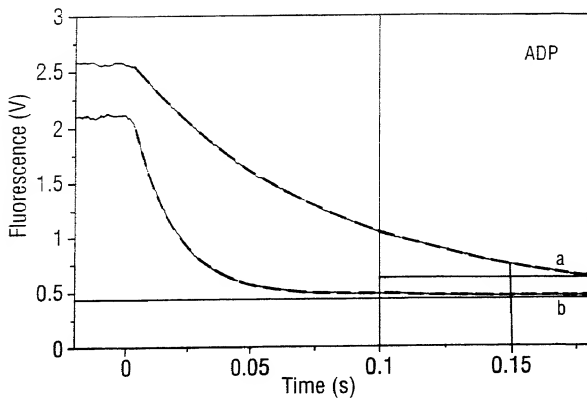
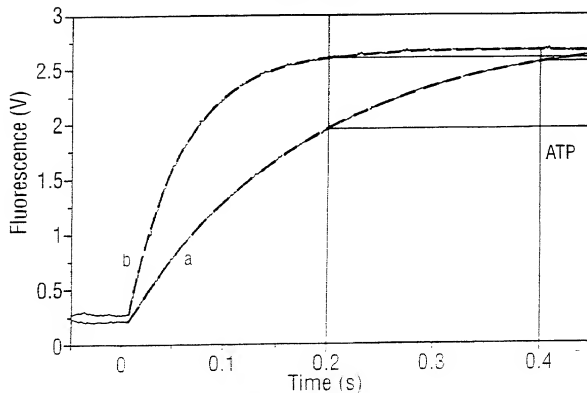
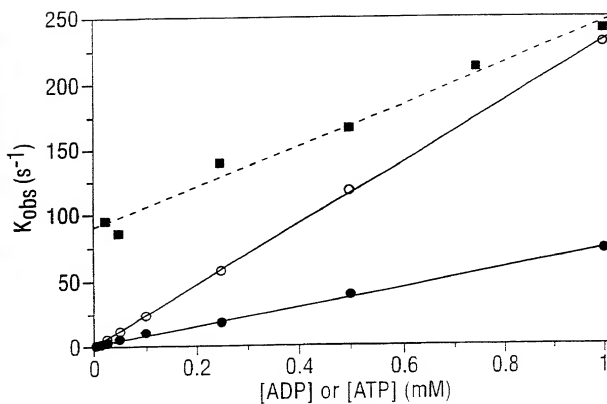


Fig. 6B



6/7

Fig. 6C



7/7

Fig. 7

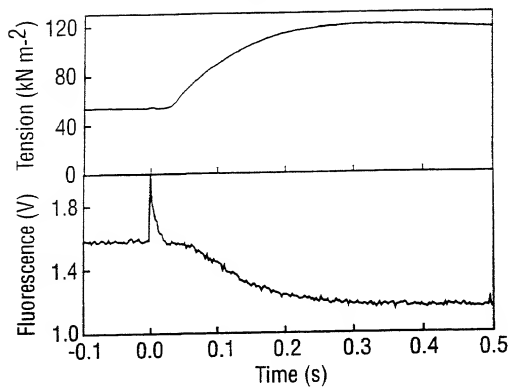
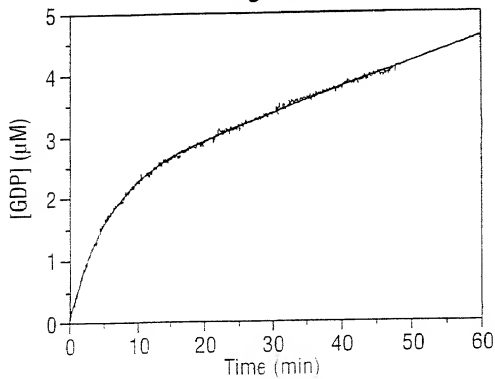


Fig. 8



DECLARATION AND POWER OF ATTORNEY FOR U.S. PATENT APPLICATION

(X) Original () Supplemental () Substitute (X) PCT () DESIGN

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and sole inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Title: ASSAYS FOR NUCLEOSIDE DIPHOSPHATES AND TRIPHOSPHATES

of which is described and claimed in:

() the attached specification, or

(X) the specification in application Serial No. NEW, filed September 25, 2001, and with amendments through _____, or(X) the specification in International Application No. PCT/GB00/01740, filed May 5, 2000, and as amended on _____ (if applicable).

I hereby state that I have reviewed and understand the content of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code, §119 (and §172 if this application is for a Design) of any application(s) for patent or inventor's certificate listed below and have also identified below any application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED
Great Britain	9910811.0	May 10, 1999	Yes

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	U.S. FILING DATE	STATUS: PATENTED, PENDING, ABANDONED

And I hereby appoint Michael R. Davis, Reg. No. 25,134; Matthew M. Jacob, Reg. No. 25,154; Warren M. Cheek, Jr., Reg. No. 33,367; Nils Pedersen, Reg. No. 33,145; Charles R. Watts, Reg. No. 33,142; and Michael S. Huppert, Reg. No. 40,268, who together constitute the firm of WENDEROTH, LIND & PONACK, L.L.P., as well as any other attorneys and agents associated with Customer No. 000513, to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

I hereby authorize the U.S. attorneys and agents named herein to accept and follow instructions from Carmacks & Ransford as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and myself. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by me.

Direct Correspondence to Customer No:



000513

PATENT TRADEMARK OFFICE

Direct Telephone Calls to:

WENDEROTH, LIND & PONACK, L.L.P.
2033 "K" Street, N.W., Suite 800
Washington, D.C. 20006-1021

Phone (202) 721-8200
Fax (202) 721-8250

Full Name of First Inventor	FAMILY NAME BRUNE	FIRST GIVEN NAME Martin Hermann	SECOND GIVEN NAME Klemens
Residence & Citizenship	CITY London	STATE OR COUNTRY United Kingdom	COUNTRY OF CITIZENSHIP German
Post Office Address	ADDRESS National Institute for Medical Research, The Ridgeway, Mill Hill London NW7 1AA, United Kingdom		

Full Name of Second Inventor	FAMILY NAME CORRIE	FIRST GIVEN NAME John Edgar	SECOND GIVEN NAME Thomas
Residence & Citizenship	CITY London	STATE OR COUNTRY United Kingdom	COUNTRY OF CITIZENSHIP British
Post Office Address	ADDRESS National Institute for Medical Research, The Ridgeway, Mill Hill London NW7 1AA, United Kingdom		

Full Name of Third Inventor	FAMILY NAME WEBB	FIRST GIVEN NAME Martin	SECOND GIVEN NAME Ronald
Residence & Citizenship	CITY London	STATE OR COUNTRY United Kingdom	COUNTRY OF CITIZENSHIP British
Post Office Address	ADDRESS National Institute for Medical Research, The Ridgeway, Mill Hill London NW7 1AA, England		

Full Name of Fourth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

Full Name of Fifth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

Full Name of Sixth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP

Post Office Address	ADDRESS	CITY	STATE OR COUNTRY	ZIP CODE
------------------------	---------	------	------------------	----------

I further declare that all statements made herein of my own knowledge are true, and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1st Inventor Martin Hermann Klemens BRUNE Date October 25, 2001
 2nd Inventor John Edgar Thomas CORRIE Date October 24, 2001
 3rd Inventor Martin Ronald WEBB Date Oct 25, 2001
 4th Inventor _____ Date _____
 5th Inventor _____ Date _____
 6th Inventor _____ Date _____

The above application may be more particularly identified as follows:

U.S. Application Serial No. NEW Filing Date September 25, 2001

Applicant Reference Number P021661US Atty Docket No. 2001_1463A

Title of Invention ASSAYS FOR NUCLEOSIDE DIPHOSPHATES AND TRIPHOSPHATES